

TISSUE AFFINITY IN AMAROECIUM. II. REAGGREGATION OF THREE PARTIAL ZOIDS INTO FUNCTIONING SIAMESE TWINS¹

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A zoid of *Amaroecium constellatum*, when macerated into fragments of tissue in the cavity of its tunic, reassembles its parts into a complete, normal zoid in a period of, approximately, five days. The capacity in tunicates that is responsible for such virtuosity of reconstitution from fragments is the fundamental property of these tissues to "recognize," grow toward, and fuse preferentially with their homologous types. It expresses itself at each level of organization: (1) The dissociated masses of each organ reassemble themselves into their respective organs, slightly smaller than the previous size in accord with the loss of some areas in maceration; (2) the reintegrated organs join together to establish their former systemic relationships; (3) the systems arrange themselves, in the recombination, according to previously existing axes of polarity. Reaggregation, therefore, is directed by the specific histogenetic nature of tissues; alignment is determined by an established axial gradient.

The facility and speed with which any detached fragment of *Amaroecium* regenerates the total organization of the zoid would lead one to expect a mass regenerative activity among the fragments following the process of maceration. Production of multiple zooids from each individual would, in this event, be the outcome. The fact that reaggregation rather than regeneration occurs indicates that affinity among tissues of common histological differentiation dominates over the regenerative process in tunicates. The character impressed on tissues, first by morphogenetic movements at gastrulation and, later, by histological differentiation, is persistent to the point of regulating the type of reaggregation in dissociated masses of tissues. In the case of *Amaroecium*, it marks all regions of an individual originating from a fertilized egg.

The present experiment was performed in order to study the capacity for reaggregation among tunicate tissues when the fragments are derived from several individuals of the same species whose contributing members, therefore, do not share a common embryonic morphogenesis and developmental environment.

MATERIALS AND METHODS

Zooids of *Amaroecium constellatum*, 12 to 18 hours old, were selected for the experiment. At this period of development the larval organs have differentiated into their adult condition and axiation. The siphons have not yet opened and the

¹ Aided by a grant from the National Institutes of Health, RG-3797(C7).

digestive tract is, therefore, free of any sand or extraneous material that might interfere with the subsequent activities of tissues and with the study of their behavior. The tissue is young adult tissue in an animal in which chordate organization is represented in its simplest expression.

Three extended zooids were divided by the sharp cleft of a micro-knife into their component branchial and abdominal halves. Two branchial regions and one abdominal region were removed from their tunics and transferred, by pipette, to a Syracuse watch glass in which two-week-old *Amaroecium* had been cultured. The older zooids were evicted from their tunics and discarded.

The three contributing members were inserted into the cavity of each host tunic where they were mildly macerated for the purpose of bringing their injured surfaces into contact with each other. While no attempt was made to arrange the members in a consistent order, they were inserted, in most cases, in two general patterns: in a direct linear order of branchial-abdominal-branchial regions, or in an abdominal-branchial-branchial order. The three halves were arranged, therefore, in tandem fashion with the abdominal member in the middle, or in the form of a Y, with the single abdominal segment forming the tail of the Y.

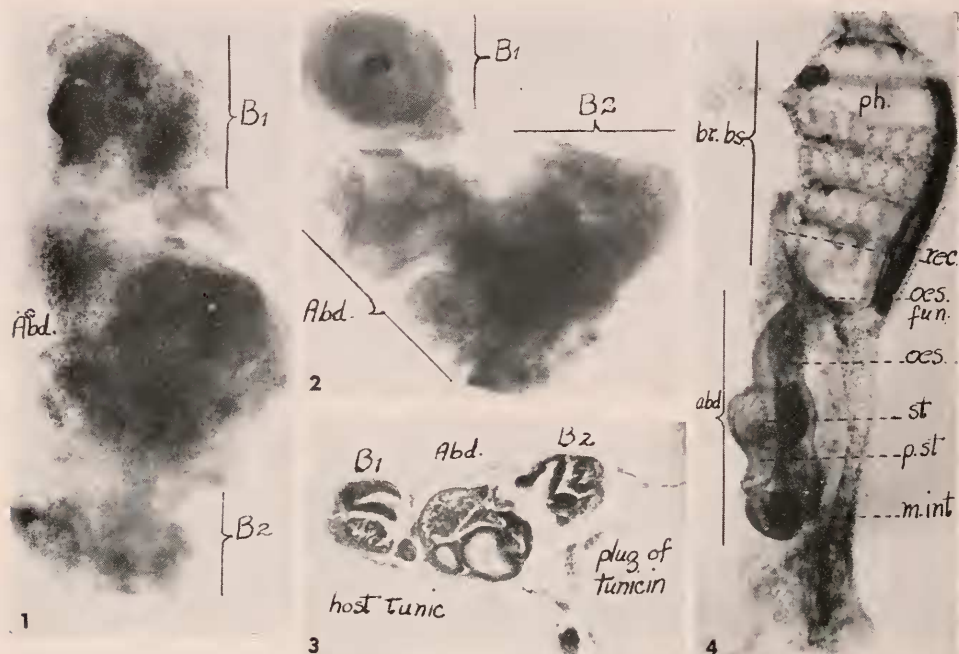
Before the members were combined in the host tunic, the caudal elements of all three contributing zooids were removed, in many cases, completely, in order to provide maximal free space within the tunic for unobstructed movements of the tissue masses. The characteristics that make the tunic of *Amaroecium* an excellent environment for experimental purposes are described in a previous paper (Scott, 1959).

After the three half zooids were macerated in the host tunic, the composites were kept intact by plugging the open tunic cavity with clumps of tunicin from the tests of the contributing zooids (Fig. 3). Numerous such combinations were made in each watch glass and the dishes were then placed in running sea water for optimal conditions of development. They were examined at frequent intervals and the series adopted for critical analysis extended from the time of combination to the reconstitution of Siamese twin organisms capable of feeding; a period covering, at the least, five days from the time of combination to the assumption of functional activity by the organisms. There were 286 such composite zooids assembled, 83 of which, 30%, were used for analytical study. Of the remaining 60%, about 30% regenerated multiple individuals because of the failure of their injured surfaces to come into contact during maceration. The remaining 30% demonstrated fusion of homologous tissues between two of the three component members and separation of the third which regenerated into a complete zooid. About 10% were fatalities. They were infested by scavenger Protozoa or consumed by small crabs in the tank.

The composite organisms were killed and fixed during this sequence at critical periods of their development. They were fixed in Schaudinn's fluid, heated to 60° C. and poured in generous quantities over the watch glass in order to obtain maximal degree of extension. The organisms for whole mounts were stained with the Feulgen technique. The serial sections were stained in Harris' hematoxylin and counterstained with triosin. All photomicrographs were taken with a Leica "Micro-Ibso" attachment, on 35 mm. Panatomic-X film.

OBSERVATIONS

The aplousobranchiate tunicates possess a systemic organization that adapts them particularly well for dissociation into regions. All structures are clearly visible and distinguishable. In its extended condition the animal can be divided easily into branchial and abdominal halves by the quick cut of a micro-knife. The branchial half of the zooid contains the pharynx with its gill clefts, endostyle and oesophageal funnel with a longer or shorter segment of oesophagus, the contiguous



FIGURES 1 and 2. Composite mass of three half zooids at time of combination.

FIGURE 3. Section of composite in the cavity of the host tunic showing the plug of tunic that holds the mass intact.

FIGURE 4. The normal zooid at the stage used for the combination. (About 90 \times .)

abd., abdominal fragment; B₁ and B₂, branchial fragments; br. bs., branchial basket; m-int., mid intestine; oes., oesophagus; oes. fun., oesophageal funnel; p-st., post-stomach; ph., pharynx; rec., rectum; st., stomach.

walls of peripharyngeal cavity, both siphons, the neural complex of ganglion and nerves, a piece of the rectal segment of the intestine at the base of the atrial cloaca, and the epidermal mantle. The abdominal half contains the remaining portion of the oesophagus, the stomach, post-stomach, mid-intestine, intestine, epicardium, pericardium and enclosed heart, body cells and enveloping epidermis (Fig. 4).

The fact that different lengths of oesophagus may be attached to both halves constitutes the only variable in tissue content in the triple combinations. It introduces no impediment to the process of reconstitution save insofar as it augments the amount of digestive tract and increases the possibility of providing the reaggregate with a second stomach.

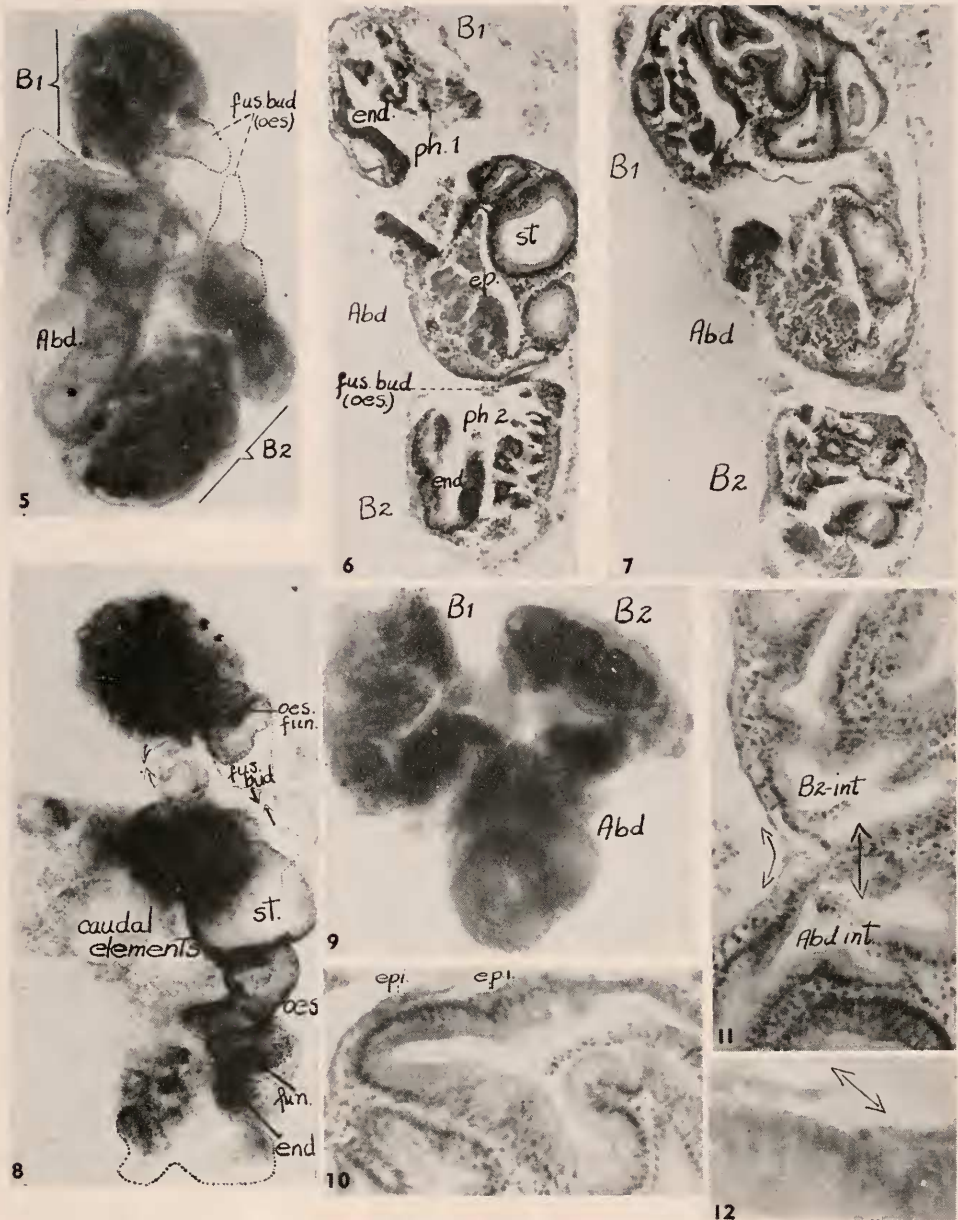


FIGURE 5. Whole mount of composite after six hours.

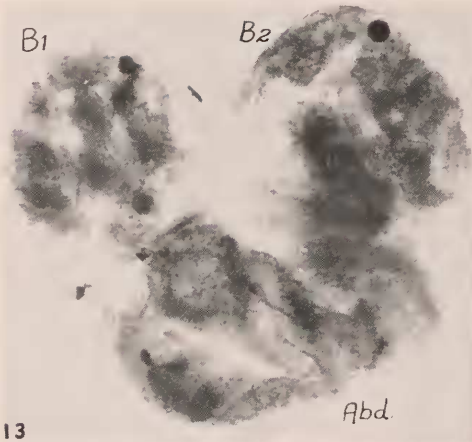
FIGURES 6 and 7. Longitudinal sections, three-hour combinations.

FIGURE 8. Whole mount after nine hours.

FIGURE 9. Whole mount of three-hour combination.

FIGURES 10, 11 and 12. Sections of three-hour combinations showing spreading of epidermis and union of abdominal and branchial intestinal fragments. (About 90 \times .)

abd. int., abdominal intestine; B₂-int., branchial intestine; end., endostyle; ep., epicardium; epi., epidermis; oes. fun., oesophageal funnel; fus. bud, fusion bud of both branchial oesophageal funnels; oes., oesophagus; ph., pharynx; st., stomach.



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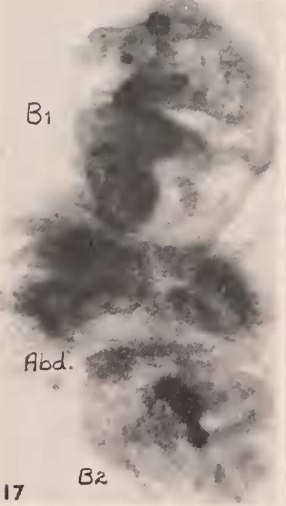
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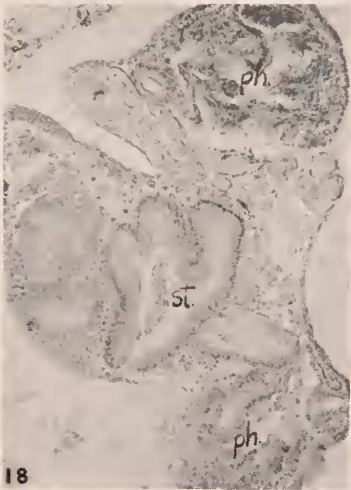
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FIGS. 13-19.

When the three contributing halves are assembled, the composite is a mass of disorganized tunicate tissues (Figs. 1, 2). By examining the living forms, the various organs can be discerned and the degree of disruption ascertained. The cilia of gill clefts and digestive epithelium continue to beat and the heart, if it is not destroyed in mincing the members together, continues to contract during and after the period of assembling and maceration. The smaller fragments of tissue that are killed by the macerating procedure, some of the body cells, and remnants of caudal elements are directed, by ciliary action, into the gaping cavities of digestive tract. The rest of this cellular detritus collects in the cavity of the tunic at the surface of, or between, the dissociated members.

Within the short period of three hours in running sea water, the composite animals show activities of fusing their multiple parts together (Figs. 6, 7, 9). The interrupted ends of epidermis commence immediately their "spreading" movements into the spaces between the fragments. The marginal cells change in shape and develop elongate processes at their free ends, the processes stretching toward similar ones on the free edges of the neighboring patches of epidermis (Figs. 10, 11, 12). This behavior of mantle is typical of *Amaroeccium* when it is reaggregating its parts into a whole after dissociation of a single individual. By such means an epidermal envelope is quickly established about the fragments of the zooid (Scott, 1959). The undisturbed mantle cells become more shallow as they spread over wider areas but they retain their optically visible histological characteristics.

The free cells in the cavity of the host tunic collect about the implanted composite and the cells in the tunic proper migrate toward its inner border, congregating there in a pseudo-epithelial layer (Fig. 6). The space between the implant and the tunic remains open during the first day of reaggregation. The space is open for two reasons; first, the animals whose parts are assembled are smaller than the host zooid whose tunic is used, in spite of the fact that three halves of the smaller zooids are used; and second, the assembled members are in a state of contraction. Their tissues slowly relax in the course of the first two days and, eventually, their joined parts fill the cavity. Upon restoration of the mantle epithelium, the implant adds its secretion of tunicin to the host tunicin and thus the mutual adoption of tunic and composite zooid is completed.

The epidermis acts more quickly than the other tissues in the process of reunion. In the early part of the first day, however, segments of digestive tract are recovering from their tight coils and they extend themselves in various directions (Figs. 6, 7, 10, 11). The injured surfaces release clusters of migrating cells that revert, in their general appearance, to a condition of pre-differentiation; that is, they lose their discernible marks of differentiation (Figs. 25, 29). Reconstitution of each organ in the digestive system is accomplished by mutual extension of corresponding

FIGURE 13. Whole mount of union one day old.

FIGURES 14, 15 and 16. Sections through one-day-old twin.

FIGURE 17. Whole mount of one-day showing linear alignment of member halves.

FIGURE 18. Section through the same type of combination.

FIGURE 19. Section through a four-day twin with fused pharynges and two stomachs. (About 90 ×.)

abd. oes., abdominal oesophagus; end., endostyle; ep., epicardium; int., intestine; m-int., mid-intestine; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.

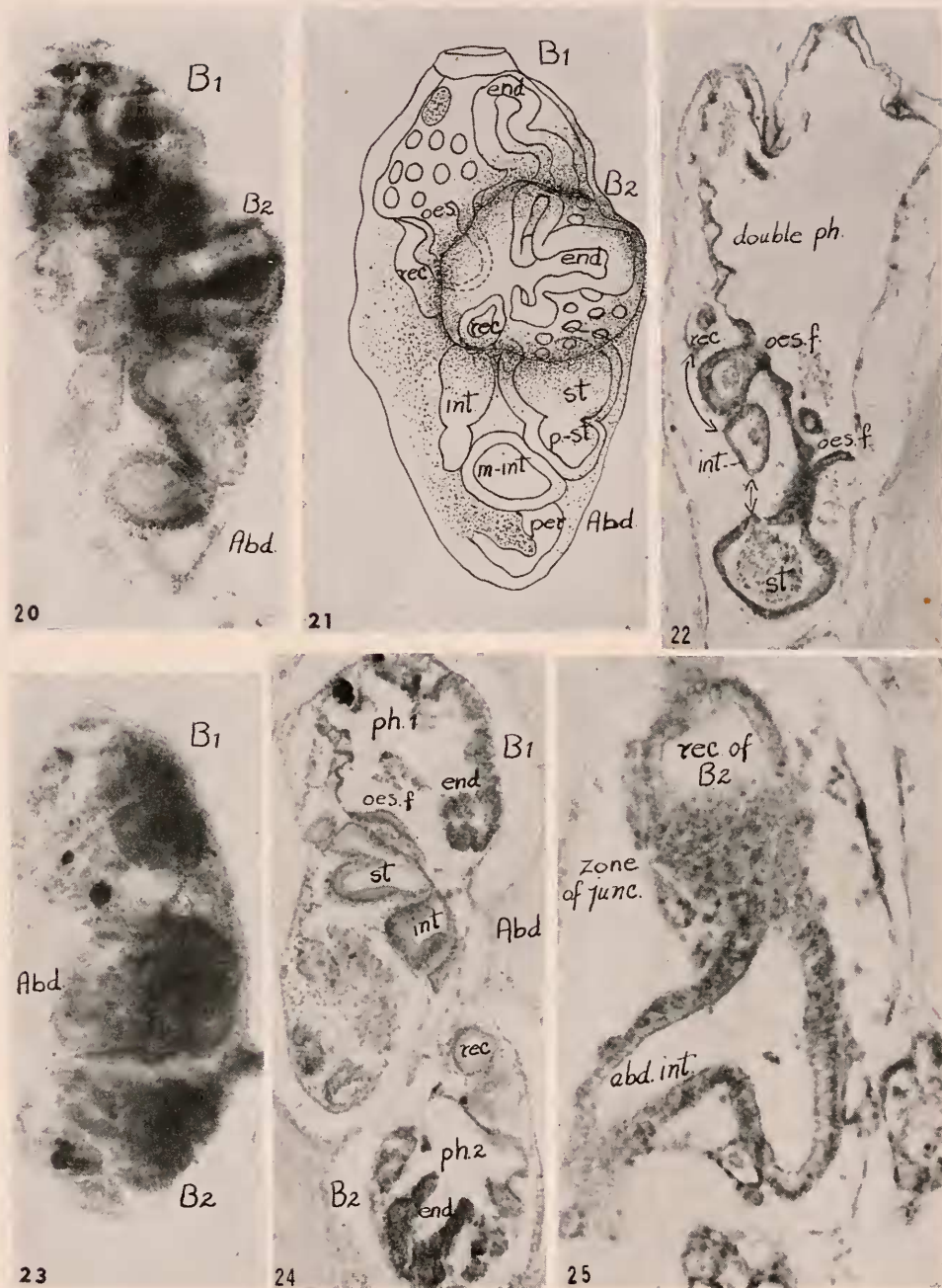


FIGURE 20. Whole mount of two-day-old twin.

FIGURE 21. Camera lucida drawing of the same specimen to show its structure.

FIGURE 22. Longitudinal section of a five-day twin with fused pharynges and one stomach.

FIGURE 23. Whole mount of a two-day-old linear twin.

clouds of cells from dissociated fragments of the same organ. They move toward each other and close all gaps. Where the gap between parts is slight, the reintegration is quickly established; where the gap is wider, reconstitution is, obviously, effected more slowly. No attempt was made in the present study to reduce the organs to inconglomerate masses of minced miscellaneous organs. This degree of maceration was accomplished in the previous study to investigate the individual tunicate's capacity for reassembling itself from its own fragments. The problem of reintegration within individual organs in these triple combinations constitutes an accessory interest and merely a prelude to the main issue. Sufficient damage is inflicted on the organs to provide surfaces of injury between and among the three half zooids, this being a condition for their subsequent union. Organ reconstitution, however, does occur in each partial zooid to the extent that the portion of enteron contained in that half is restored before the dismembered pieces establish their integrity as Siamese twins.

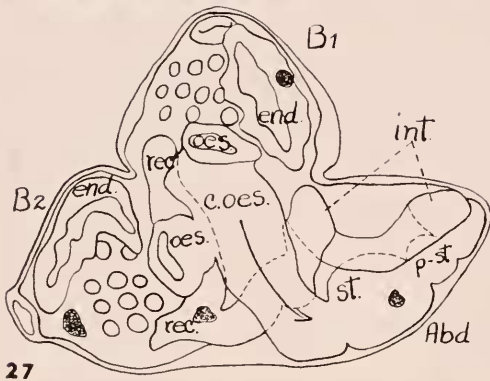
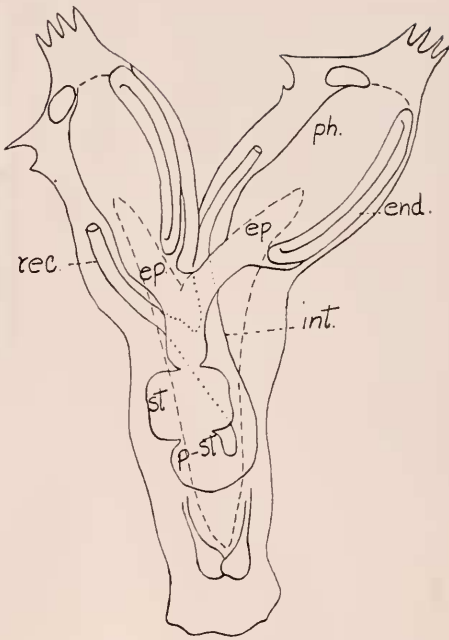
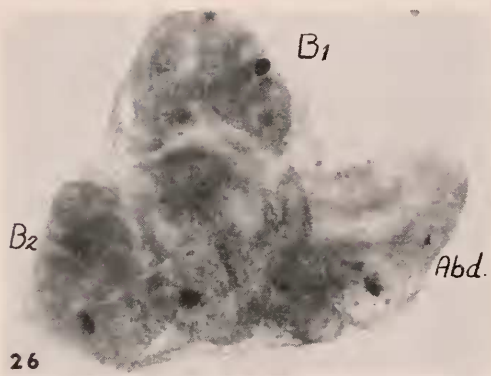
Within a day, damaged and severed sections of stomach and intestine in the abdominal half are reunited into a continuous tract, and detached areas of the two pharynges in the branchial halves are reassembled into recognizable branchial baskets with all tissues in their relative locations (Figs. 15, 16, 17, 18). The oesophageal attachment, the so-called funnel, adjusts itself according to the length of its fragment, the variations in which depend upon the exact location of the knife in cutting between pharynx and stomach. The endostyle remains in a contorted condition through this early period of pharyngeal reconstitution (Figs. 6, 15, 16). Density of cell population in the endostyle and its position along the entire length of the pharynx predispose it to rupturing in the process of even mild maceration. There may be, therefore, frequent cases in which an endostylar fragment, in combination with the contiguous pharyngo-epipharyngeal wall, lies too widely separated from the main mass of pharynx for its reaggregation in the parent member. In the detached piece, then, the process of regeneration takes over and an accessory branchial sac is produced. The accessory one may attach itself to the parent pharynx and thus endow the composite animal with one double and one single pharynx or it may form an independent zooid. (Fig. 34).

The pharynx is so expansive an organ in its normal state that its reintegration is more slowly effected than that of the more compact loop of intestine in the abdominal segment. It continues through the first day in its crumpled condition while the wholly, or partly, dissociated pieces of each branchial component reassemble themselves into two complete pharynges. If a pharynx, because of variations noted previously in the cutting, retains only the oesophageal funnel or the funnel and a very short segment of oesophagus, the free surface is converted into the typical migrating cluster of cells, the direction of which, even at this early stage, may be toward the oesophagus of the abdominal segment (Fig. 8, lower half). If the pharynx retains a longer segment of oesophagus attached to its funnel, it shows,

FIGURE 24. Longitudinal section of a linear twin of the same age.

FIGURE 25. Detail of the specimen in Figure 22, showing the zone of junction between the abdominal intestine and the branchial rectum. (About 90 \times .)

abd. int., abdominal intestine; end., endostyle; int., intestine; oes., oesophagus; oes. f., oesophageal funnel; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.



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FIGS. 26-30.

from 9 to 12 hours, a definite orientation of its "fusion" bud in the direction of the oesophageal component of the abdominal region (Fig. 6, 8, upper half).

In these early stages of reintegration the behavior of epicardium and pericardium lacks significance. The epicardium emerges gradually from its compressed condition in the abdominal members to a more extended state as the digestive loop collects its pieces together (Figs. 6, 14). In composite organisms where the epicardium has been dissociated into two or more fragments, the fragments join into a single structure. Where any portion of epicardial epithelium is lodged, it retains the capacity of differentiating into an epicardium. There may thus be several epicardia which, eventually, fuse into one (Fig. 19).

The same degree of competence marks the epicardial epithelium with respect to the formation of a pericardium and heart. The primary heart may be destroyed in the procedure of mincing; if it is destroyed, then any fragment of epicardium close to an intestinal mass may differentiate into a heart. If the primary heart is not destroyed, it continues to contract, though empty of its normal contents, through the period of reaggregation and reconstitution. By reason of such competence of the epicardial epithelium, the pericardium as a structure lacks significance in the present study of triple fusion in tunicates.

The organ-parts of the half zooids show an immediate response in all tissues, within their specific histological categories, to re-establish their integrity; first, their individual organ integrity and, second, their organ-system integrity. These activities continue throughout the first day in all areas so that, by the end of one day of development, the triple zooids are, at least, partially fused to the extent that the epidermis is actively ensheathing the entire mass within a common mantle. There may remain several narrow gaps in process of closing and the surface is anything but smooth in contour (Figs. 13-18). The contents of the mantle still present a disordered appearance. It is less chaotic, however, than that of the initial stage. Surrounding the diversity of enteric fragments are loose remnants of caudal cells, if these have not been removed when the zooids are sectioned, bits of sensory pigment, patches of epicardium, and body cells. Cellular detritus occupies the open cavities of gastric and intestinal sectors and may be found in the space between body and tunic (Figs. 14, 18, 19). "Fusion" buds of all injured surfaces of organ-parts are in process of joining their homologous parts if they have not completed the union. In most combinations the related parts of the various members are separated by wide distances, histologically speaking, and the process of reaggregation has only begun in pharynges and digestive loop by the end of the first day (Figs. 13-18).

The second and third days of development are marked by progress in reintegration of all organ parts and expansion of all areas toward their normal proportions. The epidermis, though incomplete in spots, provides the animal with a more unified

FIGURE 26. Whole mount of four-day twin.

FIGURE 27. Camera lucida drawing of the same specimen.

FIGURE 28. Diagram of the structure of Y-shaped Siamese twin in its functional stage of development.

FIGURES 29 and 30. Longitudinal sections through two regions of a Y-shaped rectal fragment. (About 90X.)

c. oes., common oesophagus; end., endostyle; ht., heart; int., intestine; oes., oesophagus; oes. f., oesophageal funnel; ph., pharynx; p-st., post-stomach; rec., rectum; sch., shuttle rectum.

appearance, and over-all expansion of tissues from their contracted state enables the body to occupy more fully the tunic cavity of the host (Figs. 20, 21, 22, 23, 24).

The oesophageal stub of each branchial sac extends farther in the direction of the homologous oesophageal stub of the abdominal member. In the composites that effect a Y-shaped union, the branchial sacs unite either separately or jointly with the stomach; that is, the two funnels may fuse and then combine with the single oesophagus of the stomach (Figs. 26, 27, 28, 29) or one funnel may engage the gastric stub and the second funnel reconstitute an independent oesophageal connection with the stomach (Fig. 22). Since the chance union of the branchial baskets in these forms ranges from a Y with widely diverging limbs through parallel alignment of pharynges to partial fusion of them, with two sets of siphons projecting at various angles from the fused mass, the paths followed by the oesophageal stumps also vary in accordance with the relative positions of the homologous parts.

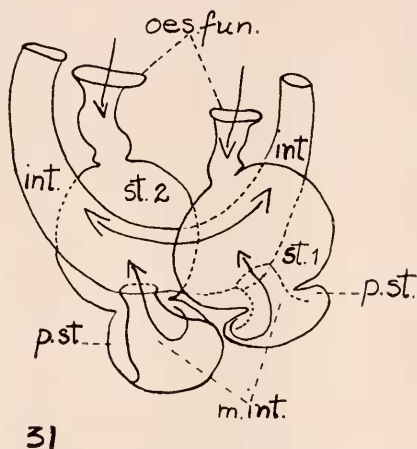


FIGURE 31. Diagram outlining the channel of traffic in a form with two stomachs and one intestine.

Each dissociation depending, as it does, on the delicate conditions of the zooid's sensitive response to cutting and the critical control of a micro-knife, within the distance of 0.1 mm., it is evident that parts of the stomach may be included in the branchial member along with the other usual constituents of that member. In such cases the partial stomach reconstitutes itself and the secondary product associates itself, at its posterior end, with the intestinal loop beyond the primary stomach. The end product in this composite possesses two stomachs, two oesophagi and one intestine. Figures 32, 33, 35 and 36 show the systemic organization of twins with two stomachs and a single intestine. Stomach 1 is the primary one, that is, the one contributed by the abdominal fragment. Stomach 2 is the secondary one which originates in a gastric remnant introduced by a pharyngeal member. The loop of intestine adapts itself to two stomachs by retaining its original relationship with the primary stomach and then establishing a secondary connection with the post-stomach of the branchial fragment, thus converting the tube into a crescent-shaped structure reaching from the double stomach toward two terminal apertures. In

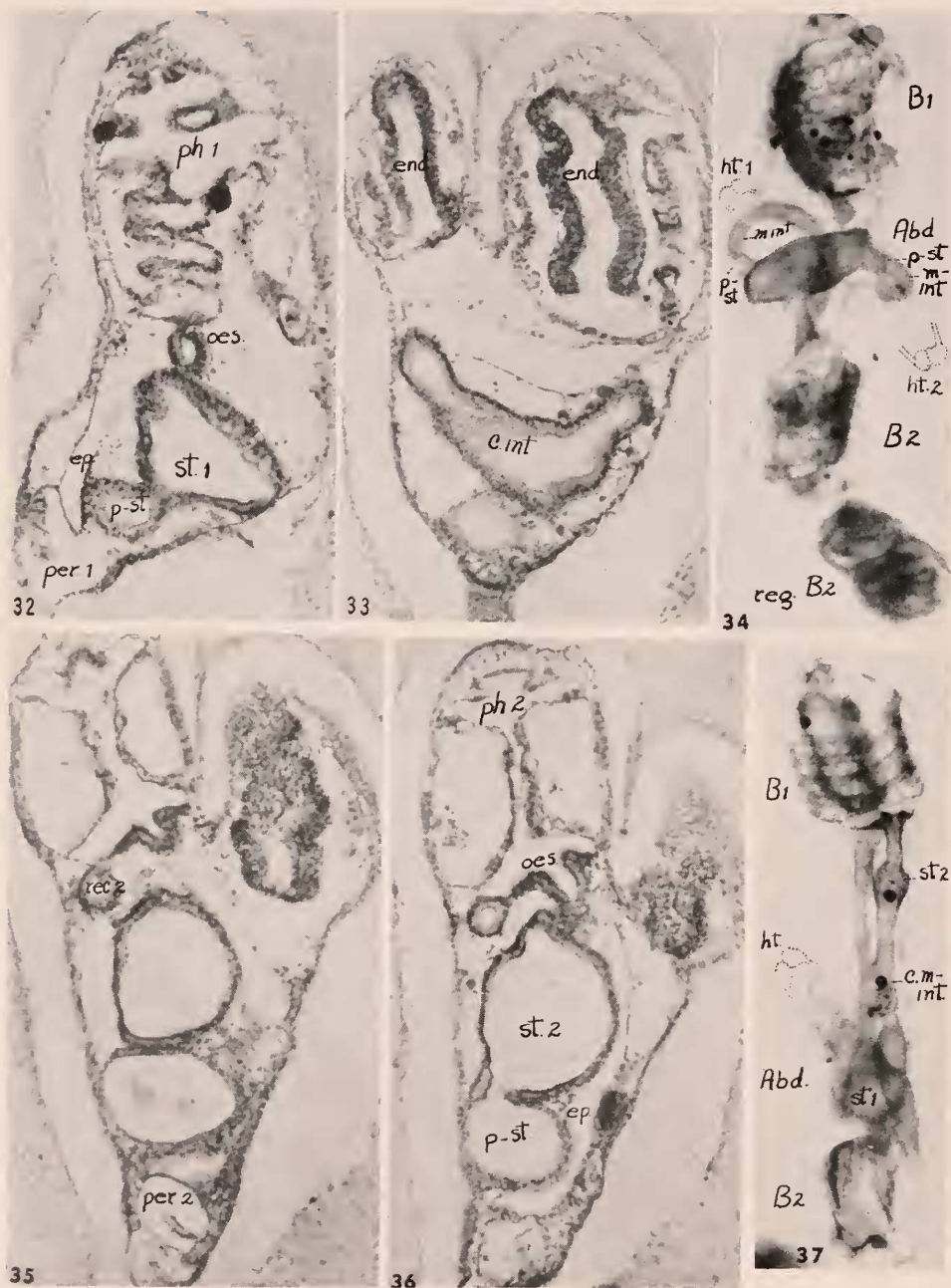
these twins, each oesophageal-stomach unit follows its independent pathway into the intestine which, then, diverges toward the rectum and anus of the two branchial components. Figure 31 is a diagrammatic reconstruction of the double gastric circuits of traffic into the common intestine and, ultimately, into the separate anus at the base of each atrial siphon. Despite the proximity of the secondary stomach to the unengaged rectal segment of the same branchial sac, the intestinal union is established between abdominal intestine and this fragment in accordance with the linear gradient of the digestive tract.

In the Y-shaped triple fusions in which a gastric fragment is not included in the branchial member, there is a single stomach serving both pharynges (Fig. 22). The alimentary tract in such twins consists, therefore, of two oesophageal funnels which relate the pharynges to the common stomach. The post-stomach, mid-intestine, and intestine may lie close to one rectal fragment and far removed from the second one. The digestive channel, in such cases, is completed without including the distant one in the union. Figure 30 demonstrates the reaction of the isolated rectal fragment to its histological situation. It produces a bud of cells which directs its growth toward the homologous rectal remnant of the sister branchial member. The bud differentiates into a shuttle channel which opens immediately adjacent to the anus in the atrial chamber of the connected alimentary tract. The traffic circuit of the enteron in these reaggregations consists of double pharyngeal chambers converging to a single oesophagus and proceeding through a common channel from that point to the anal opening in one of the branchial baskets. From that site, passage of excreta may continue directly to the atrial siphon of the branchial member involved or it may be deflected through the shuttle rectum to the siphon of the twin member on the opposite side (Fig. 30).

In many twin composites, the two pharyngeal regions are so close together that they fuse along their longitudinal axes and form a double pharynx. All structures remain duplicated, including the oesophageal funnels. In the five-day-old twin, represented in Figure 22, one funnel has and one has not made its connection with the common stomach. The intestinal segment passes behind the unconnected funnel to establish contact with the rectal fragment. The intestinal epithelial patches join with the oesophageal stub to the extent that their walls form a surface union in passing. There is functional fusing only between the intestine of the abdomen and the rectal remnant of the branchial sac. The cluster of cells reproduced in Figure 25 shows the union being established between the two intestinal fragments. Unions are also being completed between macerated fragments within the members. Stomach and mid-intestine of the same individual are reassembling themselves into an integrated whole at a point posterior to this intestinal junction between organs originating in two different individuals.

The epicardium reaggregates its parts into a unified structure by the same process of fusion of homologous parts. In Figure 19 the two constituent fragments can be seen in the act of joining together to form a single epicardial cavity. Body cells that supply nourishment for these tissue activities are concentrated at the sites of all junctions.

Zooids that form a longitudinal axis of Siamese twin, with central abdomen flanked by pharynges, present interesting patterns in morphological adaptations to tissue affinity. The stomach occupies various positions in the limited area between



FIGURES 32, 33, 34 and 35. Successive sections through a five-day-old twin with two stomachs and one intestine.

FIGURE 36. Whole mount of a five-day-old Siamese twin with a horizontal stomach.

FIGURE 37. Whole mount of a five-day-old twin with primary and secondary stomachs and a common mid-intestine. (About 90 X.)

the pharynges. Figure 37 shows the stomach lying in close proximity to one pharynx with which it unites by fusion of the pre-gastric oesophagus of the abdomen with the funnel of that pharynx. The second pharynx is farther removed from the stomach. It engages in activities of both aggregation and regeneration in establishing contact with the central digestive organs. Its oesophageal funnel regenerates a tubular channel which grows toward the mid-intestine of the primary tract with which it fuses.

The rectal fragment in each branchial donor effects association with the common mid-intestine by processes that seem to be determined by proximity between the members. The rectum lying close to the severed intestine joins it through the complementary fusion buds that characterize the reunion of all detached homologous tissues in similar macerated zooids. The more distant rectum moves by extension and cell proliferation toward the mid-intestine of the common stomach and fuses with it. This intestinal limb, like the gullet of the same pharynx, is partly the product of regeneration. The channel of traffic in this type of reaggregated *Amaroecium* now follows the course of a chiasma, with oesophageal-gastric limb crossing intestinal-rectal limb between the two pharynges (Fig. 37).

Within five days in such a twin the long oesophagus differentiates an accessory or secondary stomach at the appropriate distance from the pharynx. The two regenerated members of the composite thus establish a second series in the normal linear sequence of digestive organs without disturbing the chiasma through a common mid-intestine. Digestive traffic may proceed in either direction toward either terminal siphon from the primary stomach. Black pigment from both sensory vesicles may be seen in any region of the tract. Pigment of otolith and eye is one of the substances to be eliminated early when passage is cleared in the reconstituted digestive canal of all reaggregates of these zooids whether they be simple or multiple.

There is a single heart in the twin described, at least on the fifth day of reconstitution. It occupies the position it held in the abdominal mass. Fusion of the three masses has settled its location to a position intermediate between the two pharynges, or in the center of the digestive field.

If both branchial baskets, in a tandem arrangement of twin, are equally close to the abdominal member, another adaptation in spatial relationship occurs. The oesophageal stub of the single stomach unites with the funnel of the pharynx in its immediate field. The intestine establishes its connection with the rectal fragment of the same pharyngeal component. The stomach widens horizontally and the funnel of the twin pharynx sends its bud of cells toward the stomach on the side facing it. The corresponding posterior side of the stomach regenerates an intestinal limb which meets the bud of cells directed from the rectal fragment in the same twin atrium. A second axis of polarity is set up on this side of the complex organism in keeping with and, probably, induced by the close proximity of another pharynx to the stomach. The pharynges, in this case, form a straight angle with each other and a right angle with the common stomach. Each one assumes its proper entrance and exit according to the axial polarity that obtains between these two organs in zooids

c. int., common intestine; c. m-int., common mid-intestine; end., endostyle; ep., epicardium; ht., heart; oes., oesophagus; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.

of tunicates. The original axis of polarity is preserved between one pharynx and abdomen; a similar axis is set up between the other pharynx and abdomen. The basic pattern of spatial relationships is either retained or acquired on both sides: oesophageal funnel opens toward the dorsal region of the pharynx on its right and the intestine passes to the rectum on the left of the stomach. Identification of the primary intestine in such four-day twins is ascertained by the presence of the post-gastric and mid-intestinal valves. These have not yet differentiated in the regenerated intestine. The double axis of polarity is further marked by the development of an accessory heart formed by the epicardium in the area of the secondary intestinal loop. Figure 34 is a photomicrograph of a longitudinal twin with the stomach riding transversely between the two pharynges. The print shows a fragment of one of the pharynges which, having failed to become incorporated into its parent member, is regenerating another zooid. Such fragments form whole zooids independently of the histological activities in the reconstituting twins. A similar regenerative process occurs in fragments of a single *Amaroeccium* when they come to be located at distances from the main mass of tissues into which an organ, or region, is macerated, or when a capsule of epidermis separates any one part of an organ from the remaining fragments.

Final topographical relationships among the triple components are unpredictable: a natural consequence of the conditions necessary for inserting the pieces separately into a tunic, mincing them moderately to insure contact of injured surfaces and, finally, pressing a plug of tunicin against the pliant mass to hold it intact. There are numerous minor variations, therefore, in the final appearance of the composite organisms. There is, however, a uniform pattern of behavior among the tissues themselves that is affected only incidentally by the shifting of their major component parts.

In the abdominal member, the long segment of intestine re-establishes itself as an integrated organ from the lesser or greater number of parts into which maceration has reduced it. The valves that differentiate it into post-stomach and mid-intestine are present and all the detached patches become incorporated into a continuous alimentary tract: continuous insofar as the abdominal member is concerned; it may still be in process of effecting union with its twin homologous organs in the two branchial members, oesophageal funnel and terminal rectum. Each rectal remnant directs its fusion bud, or its proliferating bud, toward the intestinal surface. When one rectal fragment is closer to the rectal fragment of its twin pharynx than to the intestinal tissue, it selects the shorter path to its corresponding organ-part. The final association resolves itself into a bifurcated intestine (Fig. 33) or a single canal with a shuttle branch connecting one detached anus with the other integrated anus (Fig. 30). These unions are completed, or about to be effected, in 5-day aggregates but their associations are indicated by the buds of migrating cells that form at each dissociated surface of intestine, rectum and oesophagus in two- and three-day composites.

In the branchial members, the walls of branchial sacs continue to extend endostyles and patches of gill clefts into more expansive surfaces. Gaps in epidermis and respiratory epithelium are closed gradually by migratory action of the bordering cells as well as by cell proliferation proceeding at the normal tempo in the unin-

terrputed areas which, therefore, contribute cells, indirectly, toward supplying the gaps.

After five days of development, the architecture of the composite tunicate is fully established. All tissues are united according to their kinship through embryonic origin and histological differentiation. The branchial sacs, in Y-shaped or tandem association, are in functional union with the abdominal organs. The epidermis is an uninterrupted sheath enclosing the composite animal. The heart is providing the mechanical force for circulation of the body or "blood" cells through the tripartite body. The nutrition they carry has been retained through the time of reconstitution or obtained from the food passing through both oral siphons in those Siamese twins whose siphons are already functioning. In those "twins" having two hearts, both of them are in functional activity.

All early stages in both whole mounts and sections show the disordered appearance of the Siamese twins when cellular detritus is being discarded into the general tunic cavity through gaps in the epidermis. The varying amounts of tissue destroyed during mechanical dissociation determine the length of time required to clear up the tunic cavity. The black masses visible in all whole mounts of three-, four- and five-day forms are the remnants of sensory pigment that are being eliminated through the digestive tract as soon as its parts are reaggregated into a complete system. Throughout the period when the tissue components are reassembling themselves into a double monster, masses of necrotic cells are found in the lumina of all regions of the dissociated digestive tract. Ciliary movement is not interrupted either by sectioning the zooids into halves or mincing the three halves together in the host test, and the loose bits of tissue debris are drawn into all the disconnected segments of pharynx, stomach, intestine, and oesophagus. When body form is recovered, these masses are the first contents to be expelled through the atrial siphons. Sensory pigment follows and, by that time, both oral siphons are open for feeding.

The composite twins were not studied in this investigation beyond the acquisition of feeding activities. Having reassembled the various numbers of tissue components into their original organ status, and having joined the reconstituted organs of three contributing individuals into one integrated organism of Siamese twin character, with all regions aligned in the axial organization from which they were separately disjoined, the reaggregated functional zooids were considered a natural terminal stage in the present study.

DISCUSSION

The capacity of cells to recognize their own kind in a scrambled mixture; to combine selectively with them in preferential associations; and to reject any association with non-matching cells is now a well established reality. Numerous studies have contributed significantly to the mass of information available in this field. Because of the evasiveness of the issues involved in the problem of specificity of tissues, the studies tend to be descriptive rather than analytical. The question has many facets and experiments have been designed to investigate numerous aspects of it. Moscona (1960) presents one such analytical approach in his report on the significance of the extracellular material matrix (ECM) as a substitute ground substance which, in culture media, provides the cell population with a "controllable

microenvironment." He favors the hypothesis that the matrix formed in tissue cultures exerts an orienting influence on cell movements and a binding effect on the cells in their structural configurations. He, thus, assigns to the matrix the dual function of cell-bonding and transferring information.

Wilson (1908, 1910, 1911) and Galtsoff (1925) first recognized the specific attraction of dissociated cells in their classical works on sponges and hydroids. Their interest was focused on the behavior of cells dissociated and retained in the natural environment of the animals, not in tissue culture. Another report in this early period involved entire organisms at the chordate level. It is the account of Harrison and Pasquini (1930) on the Mediterranean tunicate, *Clavelina*. In their unique "piggy-back" experiment they were concerned with the demonstration of persisting polarity rather than tissue affinity. They described fusion between two segments of branchial baskets in which the axes of polarity were retained through the process of dedifferentiation characteristic of this tunicate, and through the subsequent period of reconstitution. Their line drawings of the reconstituted "monsters," interpreted in the light of the results reported in the present paper, indicate that the tissues in the grafted segment of *Clavelina* fused with matching tissues in the host zooid. The observations, however, do not refer to these morphological phenomena. A more recent study (Scott, 1959) has analyzed the specific reactions of matching tissues in a simple straightforward case of disaggregation of one complete animal, the tunicate, *Amaroeicum*, and its reconstitution into a functioning zooid. Fragments of macerated organs reassemble themselves into their respective parts and then align themselves into a complete digestive system in rigid accordance with their former axes of polarity. Within a period of five days the reconstituted zooids are feeding. In such an experiment the tissues are in fully differentiated adult condition with the exception of the stomach in which the glandular crypts have not yet developed.

The regions of the digestive tract are distinguishable from each other by reason of the simple histological patterns that characterize epithelial cells: position of nucleus, relative depth of cell ranging from deep columnar in oesophagus, stomach, post-stomach and mid-intestine, to cuboidal in intestine to squamous in pharyngeal wall. These histological tags and the unique circumstance whereby the dissociated animal can be confined within the natural (and supporting) environment of its own tunic endow *Amaroeicum* with particularly advantageous traits for the study of tissues in expressing their specificity and affinity. It is an excellent instrument for combining experiments on tissue-to-tissue attraction and the persistence of axial polarity, not merely in organ stability, but in the indestructible integrity of the individual.

These same traits are employed in the present investigation which adds another dimension to the previous study. The individual zooid, macerated in its tunic, demonstrates the behavioral patterns among tissues of similar histogenetic character which originate by processes of differentiation and organogenesis within a single embryo. The mincing together of three halves of three separate individuals, and their subsequent reassociation to form harmoniously functioning Siamese twins, reaffirms several properties shared by homologous tissues whether they originate in one embryo or find themselves in the unnatural situation of sharing a host tunic with fragments of several zooids: they join only with their own specific histological

relatives; they regulate by regenerating any deficiencies among the regions that are required for proper association of organs when one set of organs is doubled; they retain the axiation that marked their previous alignment.

The regulatory process of induction also manifests itself in some of the tandem alignments of twins. The regenerated oesophageal tube produces a secondary stomach at the site normally occupied by a stomach in the undisturbed zoid. Induction in this case does not agree with Grobstein's (1954, p. 234) definition of it as a "developmentally significant interaction between closely associated but dissimilarly derived tissue masses." The tissue is a canal of digestive epithelium regenerated from the dissociated funnel of that pharynx farther removed from the single stomach than its juxtaposed twin pharynx with whose homologous funnel the gastric oesophagus joins. The tissue is, therefore, of the same embryonic origin as the pharynx and is, in fact, derived from it. In the typical pattern of reunion of these triple components, such differentiation into regional organs does not occur. The process is a straightforward one of meeting and fusing of homologous tissues. The appearance of an incipient stomach in the unusually long oesophagus can be explained only in terms of the inductive influence of the pharynx acting on the tissue associated with it which, in the course of embryonic development, does differentiate a stomach at that site.

It may be considered an induction in the sense of Wigglesworth's (1959) theory of a "field" in which the process of differentiation follows a gradient set up or determined by the interaction of "inductor" substances with a substrate. He refers to an undifferentiated substrate, but undifferentiated in the sense of providing a ground matrix of fundamental tissue which, by reacting with the "inductor," initiates differentiation. In this case the fundamental tissue is from oesophageal epithelium, a basic type of endodermal epithelium, and it differentiates into gastric epithelium, a more specialized kind of tissue. The substances, of whatever nature they are, have effective pathways to follow in setting up a center for their activity: they may be carried in the lumen of the canal, in the body cavity or diffused through the cells themselves.

The specificity which marks all tissues in their fusion reactions conforms, also, to the principle of histological clannishness which Wigglesworth (1959) finds in the tissues of *Rhodnius*. Digestive epithelium that is differentiated into oesophageal funnels unites only with gastric oesophagus or with the superior surface of the stomach toward which the oesophageal funnel of one of the branchial baskets grows and with which it combines. If the two oesophageal funnels lie in close proximity, there is no regeneration. The two stubs fuse and the single oesophagus of the abdominal member unites with the fused pair. Regulation is determined, apparently, by proximity of matched remnants; selection, or preference, is determined not only by the fact of differentiation into digestive epithelium but, also, by reason of the degrees of differentiation along the axis that marked the tissue in its former particular niche in the digestive sequence. The fragment of rectum that is always included with the branchial basket directs its fusion bud toward the intestine of the abdominal portion, not to the stomach or oesophagus. If the rectal fragment of one branchial member is closer to the corresponding fragment in the second branchial member, then a shuttle junction is established between these two terminal sections of rectum. It serves no functional need. Elimination of waste is effected through

the intestinal loop that establishes contact between the common stomach and one of the terminal fragments. The rectal epithelium of the more distant piece, however, can satisfy its predetermined character only by uniting with its matching counterpart. Weiss and Taylor (1960) refer to this as "autonomization" or "self-organization" and they consider it as a property of a developing organism.

In this case, however, it is a property of adult tissue and totally removed from any implication of being associated with induction. It is the interaction between tissues coded, by their differentiation, to the same cues. Weiss (1947) considers the cues as chemical characteristics of contact surfaces. This response between two detached homologous fragments of rectum characterizes the behavior of all regions of the digestive tract during their reconstitution. The degree of specificity is refined to the point of region-to-region attraction within the framework of an uncomplicated system consisting of a linear series of organs of common origin and differentiated from each other only by slight variations in a continuous simple epithelium. The shuttle segment of rectum is merely one case in point. Such specific matching of tissues marks all other regions of the tract and the epicardium as well. The epicardium presents a deeper enigma in its behavior in view of the fact that its cells retain their embryonic status throughout larval and adult life until the time of budding, when each segment of epicardial tube differentiates into a complete digestive system. In the absence of differentiation beyond the basic possession of digestive epithelial potency, fragmentation might be expected to trigger the developmental mechanics of differentiation in detached patches of this tube. Constriction into segments by the epidermis initiates the process of such developmental activity in the normal period of budding. Experimental constriction of the short epicardium in early adult stage induces it, prematurely, to form a small zoid.

By mildly mincing the three half zooids into a compact mass, the epicardial sections are further disrupted into numerous disorganized fragments. The epithelia of the fragments meet, fuse, and reconstitute a common epicardium for the Siamese twins. Attraction between like tissues, therefore, dominates over the inherent capacities for both regeneration and differentiation in these tunicate tissues. Whatever the factors are that enable one tissue to recognize its kind, they operate at a higher level of determination than the (1) properties that enable tunicate fragments to reconstitute missing parts through their facile regenerative activities, and (2) the virtuosity of the epicardium in producing, with epidermal aid, a complete zoid. Specificity in tissue affinity in these primitive chordates is associated with a strong system of axial gradients and the combined set of potencies insures reintegration of a composite scramble of tunicate parts from several zooids into a monstrous but harmoniously functioning individual. The character that is impressed on tissues through the process of differentiation is persistent to the point of regulating the types of aggregation in dissociated masses of tissue whether these masses originate in one or in several individuals of the same species.

The nature of the cues which these tissues follow in re-establishing their integrity in orderly steps from organ through system to organismic level evades analysis. Wilde (1958) suggests that the characterization of coding substances may be nucleic acid or protein or both and he postulates patterns of electron flow as the chemical process whereby information may be transferred from one kind of cell to another. He locates the key to cellular differentiation and specificity at the

molecular level. Mirsky and Allfrey (1958) review various researches supporting the logical conclusion that the nucleus, specifically DNA in the nucleus, controlling as it does, through RNA, protein synthesis in the cytoplasm, must be responsible for the specificity of the cell. DNA participates in the synthesis of nucleolar RNA which may pass into the cytoplasm where RNA, in turn, may direct the synthesis of proteins that differentiate one specific kind of cell from another. The same coding, therefore, that is transferred from DNA to the cytoplasm through the medium of nucleic acids in the expression of genetic traits may be responsible for the cues that direct tissues to unite and fuse only with their own homologous types.

SUMMARY

1. Young adult zooids of *Amaroeicum constellatum* were divided into branchial and abdominal halves.
2. Two branchial halves and one abdominal half were inserted into the tunic of a two-week-old zooid from which the occupant had been evicted.
3. The three halves were mildly macerated in order to bring their injured surfaces into contact.
4. The epidermal mantle of the three half zooids, by the spreading action of its cells, enclosed the triple members in a common epidermal mantle.
5. The organ-parts in each contributing half resembled their fragments into complete organs.
6. Union of organ-parts was effected only between tissues of matching histogenetic character.
7. When the dissociated organs re-established their integrity, the organs of each half united with their homologous members in each other half.
8. The re-establishment of organs into systems followed the system of axial gradients that characterize these tunicate zooids.
9. Within five days, three halves of three zooids reaggregated their constituent parts into functioning Siamese twins demonstrating various patterns of recombination.

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